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Insights from Natural Product PHGDH Inhibitor Studies

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Abstract

Cancer cells often exhibit a reprogrammed metabolism to accommodate their higher proliferation rates. Serine is a building block for *de novo* nucleic acids synthesis, and cancer cells typically require more of it. PHGDH is the rate limiting enzyme in human serine synthesis steps and is often highly expressed in cancer. About a dozen PHGDH inhibitors have been reported, including three natural products, azacoccone E, ixocarpalactone A, and oridonin. The inhibition mechanism of most inhibitors is still lacking, and none of these inhibitors have entered clinical trials. In this commentary, we present new data on withaferin A, another natural product inhibitor of PHGDH, and discuss the inhibition mechanism by these natural products. The knowledge gained from these inhibitor studies may help guide the development of future PHGDH-targeted anticancer drugs.

Commentary

The synthetic activity of serine is significantly upregulated in several cancers [1]. *Homo sapiens* 3-phosphoglycerate dehydrogenase (PHGDH) catalyzes the rate-limiting step of serine synthesis, which converts 3-phosphoglycerate (3PG) and NAD^+ to 3-phosphohydroxypyruvate and NADH [2,3]. PHGDH inhibitors were extensively pursued recently for their use in the treatment of affected cancers [4,5]. Among the inhibitors identified, three natural products including azacoccone E [6], ixocarpalactone A [7], withaferin A and oridonin [8] were shown to effectively inhibit PHGDH enzyme activity and cancer cell growth.

Azacoccone E, an aza-epicoccone derivative from the culture of *Aspergillus flavipes*, is the first reported natural product inhibitor that was non-competitive against 3PG, but reduced the Vmax of the reaction [6]. In serine-depleted media, Azacoccone E selectively inhibited the growth of cells expressing high PHGDH, but had little inhibitory effect on PHGDH low-expressing cells. The fact that it inhibited PHGDH enzyme activity time-dependently [9] suggests that it was likely a covalent inhibitor, especially the structure of azacoccone E contains a functional group capable of forming covalent bonds [10].

Ixocarpalactone A, a natural withanolide obtained from dietary tomatillo (*Physalis ixocarpa*), not only selectively inhibited PHGDH high-expressing cells, but also exhibited anti-cancer activity in a xenograft mouse model [7]. It reduced Vmax but not Km of 3PG and NAD^+. Ixocarpalactone A contains a Michael acceptor, which can form covalent bonds with cysteine residues in a protein. At the enzyme activity level, the IC50 value of azacoccone E (9.8 ± 4.3 μM) was slightly higher than that of ixocarpalactone (1.66 ± 0.28 μM).

Through a screen of about 300 natural products, we recently reported two new PHGDH inhibitors, oridonin and withaferin A, but the study only focused on oridonin [8]. Here, we are presenting new data on withaferin A, which is a steroidal lactone derived from *Acnistus arborescens*. Withaferin A is structurally similar to ixocarpalactone A, especially the portion containing the Michael acceptor (Figure 1A). At the enzyme level, the IC50 of withaferin A (0.59 ± 0.01 μM) is similar to that of oridonin (0.50 ± 0.02 μM) (Figure 1B). These values should not be directly compared with the values for the two aforementioned inhibitors because the experimental conditions (e.g. pre-incubation time) are different. Withaferin A was shown to be a covalent inhibitor of PHGDH (Figure 2), arguing that ixocarpalactone A is also a covalent inhibitor.

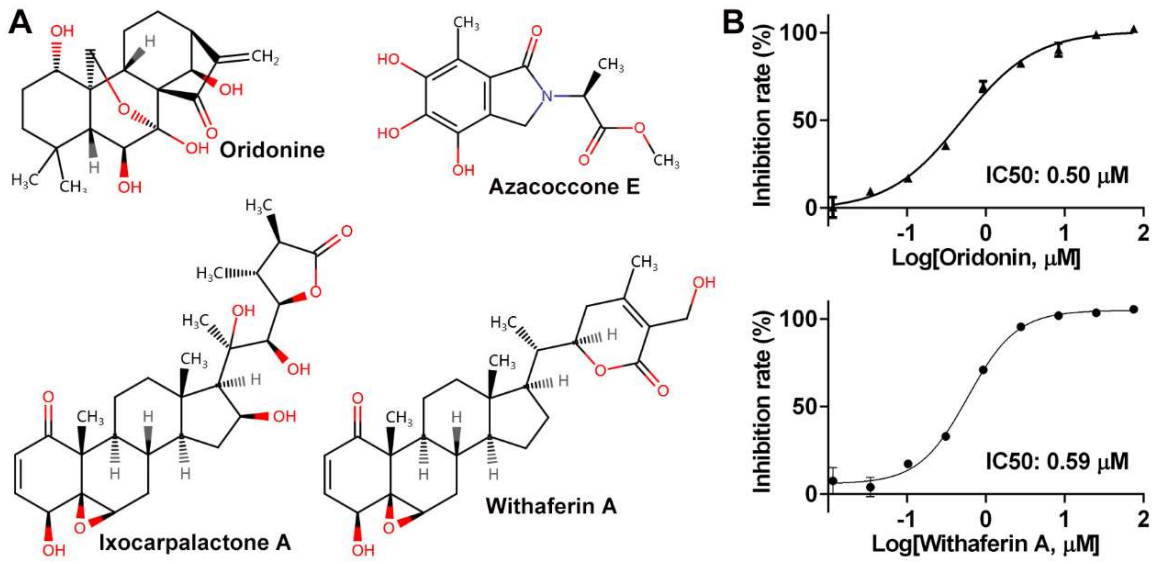

Figure 1: Chemical structures and inhibition curves of PHGDH natural product inhibitors. **A)** Chemical structures for known natural product inhibitors of PHGDH. **B)** Concentration-dependent inhibition of PHGDH by oridonin and withaferin A. The compounds were pre-incubated with PHGDH (3-314) for 2 hours before reaction.

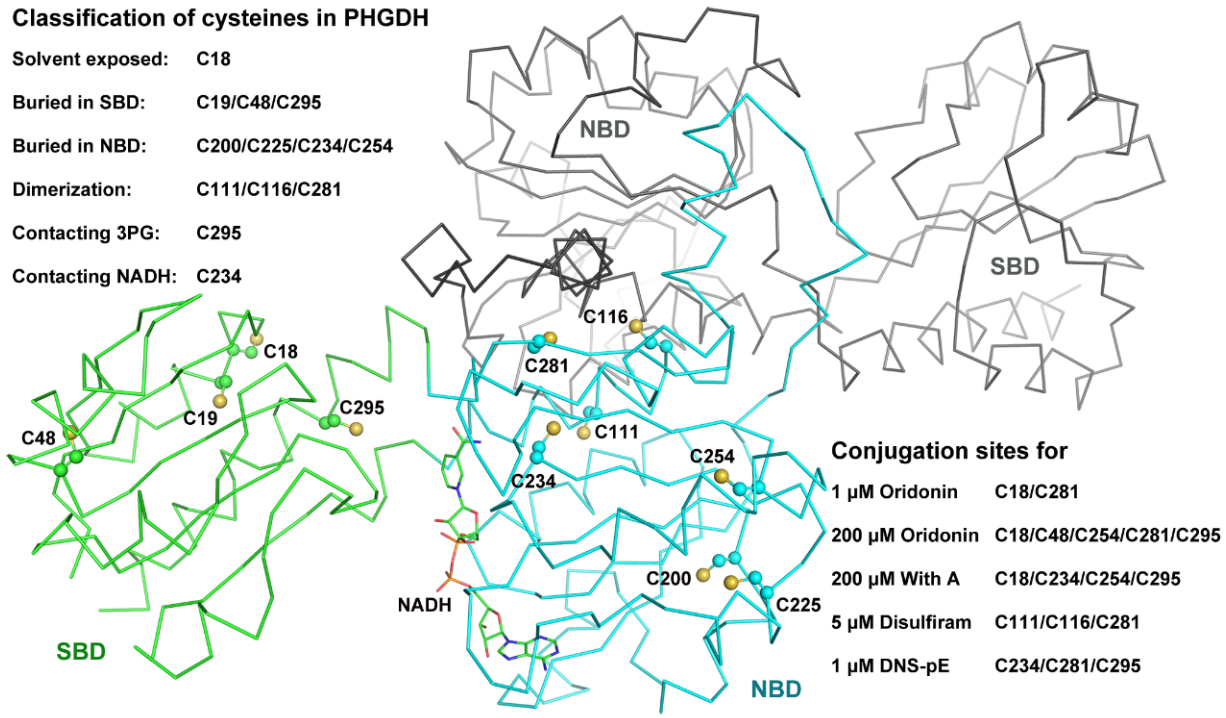

Figure 2: Locations of cysteine residues in the structure of PHGDH (3-314). The SBD and NBD of one PHGDH monomer is shown in green and cyan, respectively. Another PHGDH monomer is colored grey. Cysteine residues are shown as ball and stick structures.

Oridonin is a kaurene-type diterpenoid isolated from *Rabdosia rubescens*. Oridonin was a covalent PHGDH inhibitor with anti-proliferative activity on cancer cells [8]. Unlike azacoccone E and ixocarpalactone A, oridonin notably reduced the Km of 3PG, but barely affected the Vmax of PHGDH.

Crystal structures often provide powerful insights into the inhibition mechanism of compounds [11]. The co-crystallization of several PHGDH-inhibitor complexes was attempted, but only the complex crystal structure of PHGDH and oridonin was successfully obtained [8]. Oridonin bound in the substrate binding domain (SBD) of PGHDH, covalently linked to C18. Structural analysis revealed that a 3PG-interacting residue R54 was dislocated upon oridonin binding. R54 and C18 mutations (R54A or C18W or C18Y) that mimic the binding effect of oridonin, were shown to reduce the Km of 3PG. Therefore, oridonin binding to C18 allosterically reduced the substrate affinity of PHGDH. This newly identified allostery may help the design of allosteric drugs, which are sometimes more advantageous over orthosteric drugs [12].

Cysteine to serine mutation is often used to validate whether a site is responsible for the observed effects of a covalent inhibitor [13]. Surprisingly, oridonin inhibited C18S and WT PHGDH with similar IC50 values [8]. Isothermal titration calorimetry (ITC) also indicated that more oridonin molecules were bound per PHGDH molecule. Therefore, there are more oridonin binding sites in PHGDH, and binding to these sites played a redundant role in PHGDH inhibition.

Oridonin contains a Michael acceptor that can covalently bind to cysteine residues [14]. There are altogether 11 cysteine residues in SBD and the NAD^+ binding domain (NBD) of PHGDH (Figure 2). Only the crystallographically oridonin-conjugated C18 is solvent exposed. Other residues are mostly buried inside the protein or in the dimerization interface. Covalent modifications to C295 and C234 are expected to disrupt the binding of 3PG and NAD^+, respectively. Mass spectrometry showed that oridonin bound to multiple cysteine residues in PHGDH, and the number of sites depended on the concentration of oridonin [8]. A low concentration oridonin bound to C18 and C281. Binding to C281 is expected to disrupt dimerization and inhibit PHGDH, since the enzyme activity is dimerization dependent [15].

Except for NAD^+ competitive inhibitors, most known PHGDH inhibitors including CBR-5884, PKUMDL-WQ-2101, disulfiram, DNS-pE, and the four natural products contain covalent-binding functional groups [6-8,16-19]. A high concentration withaferin A bound to C18, C234, C254, and C295 (Figure 2), suggesting that it may disrupt binding of 3PG and NAD^+ to PHGDH. Disulfiram, an anti-alcohol drug, bound to C111, C116, and C281 [18], thus would disrupt protein dimerization. DNS-pE is likely bound to C234, C281, and C295 [19]. In a proteomic survey, multiple PHGDH cysteine residues including C18, C48, C116, C234, C254, C281, and C295 were reported to be reactive to high concentrations of small covalent modifiers [20]. Although different compounds bound to different sites, they all bound to multiple cysteine residues in PHGDH. This suggests that PHGDH is susceptible to cysteine covalent modification, and that other covalent inhibitors probably also inhibit PHGDH by binding to multiple cysteine residues.

It has been reported that oridonin degraded several cellular proteins including BCL-ABL, Glut1, MCT1, FAS [21-23]. Oridonin also induced proteasome-dependent PHGDH degradation in human cells [8]. Analysis using differential scanning fluorescence, GTA cross-linking, and size-exclusion chromatography showed that oridonin reduced the stability of PHGDH, and caused protein aggregation/precipitation [8]. These observations could explain why PHGDH was degraded in oridonin-treated cells, as cellular aggregates are often cleared by degradation machineries [24]. The other cellular targets of oridonin were probably cleared by the same aggregation-degradation mechanism.

Oridonin-induced protein aggregation may be due to two reasons. As a highly hydrophobic molecule [25], oridonin can increase the hydrophobicity of conjugated proteins, thereby promoting their aggregation/precipitation. Alternatively, binding to buried cysteine residues disrupts the protein's local folding, causing it to aggregate. At 20 μM concentration, NCT-503 (non-covalent), but not BI-4916 (NAD competitive) and CBR-5884 were shown to induced PHGDH clearance [8]. This suggests that NCT503 (at 20 μM) is 'on target' in cells, and that covalent-binding is not essential for PHGDH clearance. The aggregating-degrading ability of oridonin may be shared by other natural product PHGDH inhibitors, but more investigations are needed.

While testing whether cancer mutants were sensitive to oridonin, many cancer mutants (6 out of 8) were found to have impaired enzyme activity [8]. This seems to contradict the notion that cancer cells require more serine synthesis [26-28]. Several studies have demonstrated non-metabolic roles of PHGDH in different cancers [29-31]. For example, PHGDH interacted with the translation initiation factors eIF4A1 and eIF4E to facilitate the assembly of the translation initiation complex, thereby promoting the development of pancreatic cancer [30]. PHGDH was also reported to interact with and stabilize FOXM1 at the protein level, promoting the proliferation, invasion, and tumorigenicity of glioma cells [32]. Therefore, rather than focusing on inhibiting enzyme activity, it may be more desirable to develop PHGDH-degrading inhibitors, which may have a broader application in PHGDH-dependent cancers.

In summary, four natural products were shown to directly engage and inhibit PHGDH. However, the inhibition mechanisms are quite different. Unlike Vmax type inhibitors azacoccone E and ixocarpalactone A, oridonin reduce the affinity of the substrate 3PG. The crystal structure indicates

that a critical substrate-binding residue was relocated upon oridonin binding. Furthermore, multiple cysteines in PHGDH were prone to covalent binding by different covalent inhibitors. Selective clearance of cellular PHGDH may represent a new strategy for the treatment of PHGDH-dependent cancers. The insights generated may facilitate the mechanistic study for other known inhibitors and the development of new PHGDH inhibitors.

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Conflict of Interest

The authors declare no conflict of interest.

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